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13. ABSTRACT (Maximum 200 words) Hibernating mammals provide a unique system for identifying molecules important in regulating metabolism, body temperature, and sleep. In a state of deep hibernation, body temperature is only a few degrees above 0°C and heart rate can be as low as 3-10 beats/min, compared to 200-300 beats/min when the animal is awake and active. We have identified genes that are responsible for regulating the physiological characteristics of hibernation in the thirteen-lined ground squirrel <i>Spermophilus tridecemlineatus</i> . In our screen we identified a new protein, CADp44. Based on our findings using yeast-genetics and amino acid sequence homology, we showed that CADp44 is a regulatory sub-unit of the 26S proteasome. Over the final two years of the project we have: 1. Isolated additional cDNAs from the heart that are specific for entry into hibernation and long-term maintenance of the hibernating state. 2. Confirmed the specificity of differentially expressed mRNAs and determined the sequence of cDNA fragments that have been shown to be specifically up-regulated or down-regulated during these processes. 3. Begun to develop physiological assays based on this information.				
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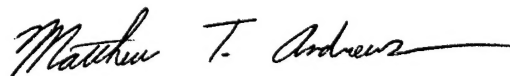
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REPORT TITLE: A New Regulatory Protein Expressed in Hibernating Mammals

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SUBMITTED FOR PUBLICATION TO (applicable only if report is manuscript):

Sincerely,



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Enclosure 3

FORECAST EXPENDITURE REPORT
(IMPORTANT!>>>YOU MUST REPLY TO RECEIVE ADDITIONAL FUNDING<<<)

The research agreement identified below is under consideration for additional funding. The contemplated funding, resulting in an extension to the performance period of the agreement, is consistent with the terms and conditions of the agreement. Before a decision can be made to provide the additional funding, the following information is needed: (i) an accounting of costs incurred to date and (ii) a projection of financial needs for the period

b. FINAL PROGRESS REPORT

A New Regulatory Protein Expressed in Hibernating Mammals

Matthew T. Andrews, P. I.

Statement of the problem studied

Hibernating mammals provide a unique system for identifying molecules important in regulating metabolism, body temperature and sleep. In a state of deep hibernation, body temperature hovers a few degrees above 0°C, oxygen consumption holds at 1/30 to 1/50 of the aroused condition and heart rate can be as low as 3-10 beats/minute, compared to 200-300 beats/minute when the animal is awake and active. We have begun to characterize the genes responsible for regulating the physiological characteristics of hibernation in the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, and are using genomic strategies to identify new genes that control the hibernating phenotype.

Among mammals, the process of hibernation is found in a limited number of species that span a wide range of taxa. Hibernation is seen in certain species of rodents, carnivores, insectivores, bats and even primates. Since the majority of species within these groups do not hibernate, it has been proposed that hibernation results from the differential expression of existing genes rather than the evolution of new genes unique to the hibernating species. This hypothesis could explain why certain species of ground squirrels hibernate, yet other closely related squirrel species do not. If hibernation is controlled by the differential regulation of existing mammalian genes, then identification of these genes could provide insight in to how humans may adapt to similar environmental extremes.

Summary of the most important results

- **Genomic approaches to study hibernation.** We have focused our efforts on the regulatory aspects of hibernation by identifying genes that are differentially expressed during the hibernating state. We have used a PCR-based gene expression screen to isolate cDNAs of genes showing increased levels of expression during hibernation in both the heart and hypothalamus of thirteen-lined ground squirrels. This subtractive approach has yielded cDNAs for several differentially regulated genes, some of which are described below. Soon we will begin screening microarrays of summer active and hibernating animals to identify and isolate additional differentially expressed sequences.
- **Genes we have identified.** This project began in 1996 and has resulted in the isolation of cDNAs coding for proteins of both known and unknown function. The first year we identified a novel mammalian ATPase, **CADp44**, that we later determined to be the sixth (and probably final) AAA domain-containing regulatory subunit of the 26S proteasome. In 1997 we found that genes encoding **pancreatic triglyceride lipase (PL)** and **pyruvate dehydrogenase kinase isozyme 4 (PDK-4)** are up-regulated in the heart when hibernation begins, and that steady-state levels of both mRNAs remain high while metabolism and body temperature are greatly depressed. Recently we have identified increased expression of the protease inhibitor **calpastatin** both in heart and in skeletal muscle. Induction of protease inhibitors, and/or down-regulation of muscle proteases, could explain the greatly reduced muscle disuse atrophy normally seen in animals that have been inactive for periods of several months.
- **Characterization of gene products.** Functional genomics has permitted us to construct and test model biochemical pathways specific to the hibernator (Figure 1). Involvement of PDK-4 and PL in the intermediary metabolism of the ground squirrel heart contributes to the observed respiratory quotient (RQ) of 0.7 during hibernation. RQ is a unit-less value representing the moles of CO₂ respired per moles of O₂ consumed. A value of 1.0 indicates combustion of carbohydrate; however, an RQ of 0.7 indicates that fat is the major substrate for energy production.

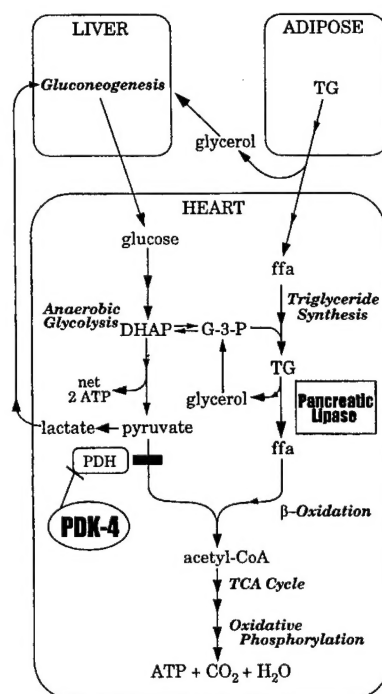


Fig. 1. Model showing the metabolic involvement of pyruvate dehydrogenase kinase isozyme 4 (PDK-4) and pancreatic lipase in the heart of a hibernating thirteen-lined ground squirrel. Names of metabolic pathways are shown in *italics*. Arrows with a single arrowhead indicate a single reaction. Continuous arrows with two or more arrowheads indicate multi-step pathways. Abbreviations: ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; ffa, free fatty acid; G-3-P, L-glycerol 3-phosphate; PDH, pyruvate dehydrogenase; TCA cycle, tricarboxylic acid cycle; TG, triglyceride.

- **PDK-4.** The fuel selection of fat over carbohydrate seen during hibernation also happens in starvation, when conservation of glucose for utilization by the brain is essential for survival and is controlled by the PDK-4-mediated phosphorylation of pyruvate dehydrogenase. Since hibernating ground squirrels can survive 5-6 months without feeding, mechanistic insight into the activation of genes such as PDK-4 can provide a better understanding of how nutrient-gene interactions influence an animal's capacity to cope with the effects of starvation. We are currently investigating this mechanism by studying hormonal regulation of PDK-4 in hibernating animals.
- **PL.** The gene for pancreatic lipase (PL) is up-regulated in the heart throughout the hibernation season. cDNA sequencing indicates that PL from heart tissue shares 85% amino acid identity with the unprocessed form of human pancreatic lipase. PL is normally expressed exclusively in the pancreas, but when expressed in the hibernating heart it liberates fatty acids from triglycerides at temperatures as low as 0°C. This low-temperature lipolysis provides a steady supply of fuel for the working heart during periods of near-freezing torpor. Recently we detected expression of an unusual chimeric retroviral-PL mRNA in white adipose tissue. We are currently investigating the evolutionary and adaptive implications of

this novel expression of a low-temperature lipase in the main fat storage depot during hibernation.

- **Low-temperature biocatalysis.** The potentially wide functional temperature range of ground squirrel enzymes during hibernation is attractive due to various practical uses and versatility of handling. For example, purified heart PL could be used to hydrolyze triglycerides at temperature ranges that normally cannot be controlled (e.g. outdoor environments). Since greater than 90% of dietary fat is in the form of triglycerides, PL has potential as a food additive to change palatability and emulsifying characteristics of low cost natural fats to resemble more desirable and less available fats at temperatures that prevent spoilage. Another application would be as a component of household laundry detergents to effectively remove grease and lipid-based food stains in cold water. Recently, recombinant PL has been expressed in yeast and shows the same low-temperature activity as that seen in extracts from ground squirrel hearts.
- **Post-genomic significance.** Advances in the areas of organ preservation, anesthesia and sleep disorders could potentially result from a better understanding of the molecules that regulate mammalian hibernation. Neuropeptides, analogous to sleep peptides, could be far more stable and possibly more abundant in a hibernating animal due to the length of torpor. Other examples may include endorphin-like peptides, similar to β -endorphins, that lower body temperature and induce analgesia over the entire body. Unlike the short-term effect of a β -endorphin (which disappears within a few hours), corresponding peptides in hibernating animals could have extremely long half-lives or be synthesized continuously. Considering the various physiological extremes of the hibernating phenotype, uncovering the function of genes involved in mammalian hibernation has the potential to greatly increase our understanding of human responses to related stresses such as hypoxia, hypothermia and starvation.

List of all publications and technical reports

Published articles:

- Bauer, V.W., Swaffield, J.C., Johnston, S.A., and Andrews, M.T. (1996) CADp44: A novel regulatory subunit of the 26S proteasome and the mammalian homolog of yeast sug2p. *Gene* **181**, 63-69.
- Andrews, M.T., Squire, T.L., Bowen, C.M., and Rollins, M.B. (1998) Low-temperature carbon utilization is regulated by novel gene activity in the heart of a hibernating mammal. *Proc. Natl. Acad. Sci. USA* **95**, 8392-8397.
- Brown, J.A., Chua, S.C., Liu, S.M., Andrews, M.T., and Vandenberg, J.G. (2000) Spontaneous mutation in the db gene results in obesity and diabetes in CD-1 outbred mice. *Am. J. Physiol.* **278**, R320-R330.

Squire, T.L. and Andrews, M.T. (2000) Genetic control of carbon utilization during hibernation: mechanistic considerations, in "Life in the Cold" (G. Heldmaier, S. Klaus, M. Klingenspor, Eds.) pp. 325-337, Springer-Verlag, Berlin.

Bauer, V.W., Squire, T.L., Lowe, M.E., and Andrews, M.T. (2000) Novel expression of pancreatic lipase in white adipose tissue confers low-temperature lipolysis in a hibernating mammal. *Am. J. Physiol.*, in press.

Abstracts:

Andrews, M.T., Bauer, V.W., and Swaffield, J.C. (1996) CADp44: A New Regulatory Protein Expressed in Hibernating Thirteen-lined Ground Squirrels. Tenth International Hibernation Symposium, June 30-July 6, 1996, Cradle Mountain, Australia.

Andrews, M.T. (1996) Developing *Xenopus* embryos as a living test tube for transcription factor function. Genetics Society of Australia 43rd Annual Conference, July 7 - 11, 1996, Melbourne.

Bauer, V.W. and Andrews, M.T. (1996) CADp44: a novel protein expressed in hibernating ground squirrels. Response and Adaptation to the Environment, October 24 -26, 1996, Research Triangle Park, NC.

Bowen, C.M. and Andrews, M.T. (1996) A temperature-dependent study of lipase function in hibernating thirteen-lined ground squirrels. Response and Adaptation to the Environment, October 24 -26, 1996, Research Triangle Park, NC.

Andrews, M.T., (1998) Genes controlling low-temperature carbon utilization in a hibernating mammal. Biomolecular Signaling, Energy Transfer and Transduction Processes; Physiology and Performance, May 4-6, 1998, Cashiers, North Carolina.

Squire, T.L., Bauer, V.W., and Andrews, M.T. (1999) Role of hibernation-specific gene activity in controlling low-temperature metabolism. Hibernation and Adaptations to the Cold, May 20-22, 1999, Estes Park, Colorado.

Bauer, V.W., Squire, T.L., Lowe, M.E., and Andrews, M.T. (1999) Novel expression of pancreatic lipase during hibernation in the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*. Hibernation and Adaptations to the Cold, May 20-22, 1999, Estes Park, Colorado.

Buck, M.J., Squire, T.L., and Andrews, M. T. (1999) Differential expression of genes encoding mitochondrial proteins in cardiac and skeletal muscle of the hibernator, *Spermophilus tridecemlineatus*. Hibernation and Adaptations to the Cold, May 20-22, 1999, Estes Park, Colorado.

Squire, T.L., Bauer, V.W., Buck, M.J., and Andrews, M.T. (1999) Genetic control of low-temperature metabolism in a hibernating mammal. Fifth International Congress of Comparative Physiology and Biochemistry, August 23-28, 1999, Calgary, Alberta, Canada.

Participating scientific personnel

GRADUATE STUDENTS

Erika Bartolucci – Masters of Genetics, May 1999
Vernon Bauer - Ph.D. in Genetics, December 1999
Teresa Squire - Ph.D. in Genetics, expected May 2001
Michael Buck
Jayita Guhaniyogi

UNDERGRADUATE RESEARCH PROJECTS DIRECTED

Mikie Dawson, B.S. 1998
Amy Phillips, B.S. 1998
Michael Fielder, 1998-99
Luis Miguel Gonzalez, 1999-2000

Report of inventions

none